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Immunopathology and Infectious Diseases

On the Etiology of Type 1 Diabetes

A New Animal Model Signifying a Decisive Role for Bacteria Eliciting an Adverse Innate Immunity Response

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The cause of type 1 diabetes (T1D) remains unknown; however, a decisive role for environmental factors is recognized. The increased incidence of T1D during the last decades, as well as regional differences, is paralleled by differences in the intestinal bacterial flora. A new animal model was established to test the hypothesis that bacteria entering the pancreatic ductal system could trigger β-cell destruction and to provide new insights to the immunopathology of the disease. Obtained findings were compared with those present in two patients dying at onset of T1D. Different bacterial species, present in the human duodenum, instilled into the ductal system of the pancreas in healthy rats rapidly induced cellular infiltration, consisting of mainly neutrophil polymorphonuclear cells and monocytes/macrophages, centered around the pancreatic ducts. Also, the islets of Langerhans attracted polymorphonuclear cells, possibly via release of IL-6, IL-8, and monocyte chemotactic protein 1. Small bleedings or large dilatations of the capillaries were frequently found within the islets, and several β-cells had severe hydropic degeneration (ie, swollen cytoplasm) but with preserved nuclei. A novel rat model for the initial events in T1D is presented, revealing marked similarities with the morphologic findings obtained in patients dying at onset of T1D and signifying a decisive role for bacteria in eliciting an adverse innate immunity response. The present findings support the hypothesis that T1D is an organ-specific inflammatory disease. ***(Am J Pathol 2012, 181: 1735–1748; http://dx.doi.org/10.1016/j.ajpath.2012.07.022)***

Our understanding of the etiology of type 1 diabetes (T1D) remains limited and originates to a large extent from two animal models: the nonobese diabetic mouse and the BioBreeding-diabetes prone rat.[1] In both models a progressive T-cell–mediated destruction of the β-cells occurs; however, the immunopathologic findings reveal limited similarities with the human disease.[2–5] In human pancreatic specimens, insulitis is discrete, affects only a few islets, and is heterogeneously distributed within the gland. In a recent meta-analysis, insulitis was reported in only 29% of patients with onset between 15 and 39 years of age and with a disease duration of <1 month.[6] At the time of diagnosis, autoantibodies were only present in approximately 70% to 80% of affected patients.[7] Likewise, attempts to prevent disease progression with immunosuppression[8–11] or immunointerventions[12–14] cause no or only transient preservation of β-cell function.

The fact that the exocrine pancreas gets affected in patients with T1D is underappreciated, and several studies have found autoantibodies in the exocrine cells before the onset of T1D.[15–18] Mild to moderate exocrine pancreatic insufficiency is an early event in T1D,[19] and a substantial reduction (32%) in pancreatic volume is already present 3 to 4 months after onset.[20] Also, in the classic report by Gepts,[4] lesions of the acinar tissue were reported to occur frequently in patients with recent onset of

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T1D. The findings comprised mostly focal or diffuse lesions of acute pancreatitis with accumulation of leukocytes, often centered around the excretory canals.[2–5] In a more recent study of patients with long-term T1D, 40% had periductal fibrosis and 60% of cases had periductal fibrosis that extended to the interlobular pancreas.[21] Collectively, these observations suggest that the injurious process that causes T1D affects both the exocrine and endocrine components of the pancreas and challenge the view that T1D is a β-cell–specific autoimmune disease.

The low concordance rate for the development of T1D in identical twins and the current rapid increase in incidence of T1D argue against a decisive role for genetic factors. Notably, there is a close to sixfold gradient in the incidence of T1D between Russian and Finland Karelia, although the population is homogenous and the predisposing HLA genotypes are equally frequent.[22] In addition, children born in Finland by immigrants from Somalia, a low incidence country for T1D, acquire the same risk for T1D as the native Finish population.[23] On the basis of these and similar observations, it is generally assumed that environmental factors may act as triggers of T1D. For decades different enteroviruses have been implicated in the pathogenesis of T1D[24]; however, evidence of causality remains missing.

Bacterial colonization of the infantile gut is influenced by environmental factors and has changed markedly in developed countries during the last decades.[25] Interestingly, the increased incidence of T1D[26] and the difference in incidence of T1D in Sweden, Italy, and Africa[26–28] are paralleled by reported frequencies of intestinal *Staphylococcus aureus*.[29–32] Bacteria entering the ductal system of the pancreas would be exposed to the pancreatic juice–containing substances, with marked antibacterial effects initiating release of bacterial components, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), and various toxins. Notably, these substances have been implicated in the etiology of neurogenerative diseases and neural cell death because they stimulate microglia to produce proinflammatory cytokines (IL-1b, IL-6, tumor necrosis factor-α), nitric oxide, and reactive oxygen species, causing significant cell death in neighboring neural cells.[33]

The present study was conducted to establish an animal model for the initial events in T1D to test the hypothesis that bacteria entering into the ductal system of the pancreas could elicit an adverse innate immunity response. Different bacterial species present transiently or continuously in the human duodenum were instilled into the ductal system of the pancreas in healthy rats. To examine the clinical relevance of the experimental model, obtained findings were compared with those present in the pancreases of two patients dying at onset of T1D.

Materials and Methods

Ethics

All work involving human tissue was conducted according to the principles expressed in the Declaration of Helsinki and in the European Council's Convention on Human Rights and Biomedicine. Consent for organ donation (for clinical transplantation and for use in research) was obtained from the relatives of the deceased donors by the donor's physicians and documented in the medical records of the deceased patient. The study was approved by the Regional Ethics Committee in Uppsala, Sweden, according to the Act Concerning the Ethical Review of Research Involving Humans (2003:460; permit number Dnr 2009/043, 2009/371).

The animal experiments were in accordance with the Swedish Animal Welfare Act (SFS 1988:534) and The Swedish Animal Welfare Ordinance (SFS 1988:539) both in agreement with directives 86/609/EEG and 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. The ethical application was approved by the Uppsala Laboratory Animal Ethical Committee (permit number C153/10).

Human Pancreatic Specimens

A total of 23 human pancreases were included in the study. Two pancreases were obtained at the onset of T1D (patients 1 and 2), and the remaining 21 were collected consecutively from multiorgan donors of similar age and without any known pancreatic disease in the time span between the two T1D patients. The latter donors were aged 29 to 40 years (mean $\pm$ SD age, 35.6 $\pm$ 0.7 years).

Patient 1

A 29-year-old, previously healthy male (HLA-A24, HLA-B39, HLA-DR4) walked to the emergency department of the local hospital after 2 days with increased thirst and nausea. At arrival, patient was fully conscious, oriented (Glasgow Coma Scale score of 15), and vomiting. Laboratory test results were as follows: B-glucose, 46 mmol/L; B-pH 6.92; P-HCO_3^-, <3; B-hemoglobin, 187 g/L; and P-sodium, 125 mmol/L. Rehydration was initiated together with an i.v. bolus of short-acting insulin, 10 IU, which was repeated after 3 hours, at which time the B-glucose level was 31 mmol/L. Physical status deteriorated during transfer to another hospital. At admittance 5 hours after the initial contact, he was unconscious and suddenly stopped breathing. The patient was intubated and given norepinephrine. Laboratory tests revealed further derangements: P-Na, 168 mmol/L; P-potassium, 4 mmol/L; B-hemoglobin, 115 g/L: C-reactive protein, 69 mg/L; and P-amylase, 82 μkat/L. Computed tomography (CT) revealed massive brain edema and a suspected massive subarachnoid hemorrhage. The patient was referred to the Department of Neurosurgery, Helsinki University Hospital. The brain edema increased, and the patient developed total brain infarction. The death of the patient was considered the result of a series of complications associated with the recent onset of T1D.

Patient 2

The patient was a 40-year-old, previously healthy male (HLA-A23/24, HLA-B27/44, HLA-DR4/7) with a history of

Table 1. Bacteria and Culture Conditions

Bacterial species	Strain	Origin	Culture media	Growth phase	Viable count
E. faecalis	B1008314	Blood	BHI broth	Late stationary phase	10^9 CFU/mL
E. coli	S0705198	Peritoneal fluid	BHI broth	Late stationary phase	10^9 CFU/mL
E. faecalis	B1008314	Blood	BHI broth with 70% CMRL-1066 and 5% FBS	Early stationary phase	10^9 CFU/mL
E. coli	S0705198	Peritoneal fluid	BHI broth with 70% CMRL-1066 and 5% FBS	Early stationary phase	10^9 CFU/mL
S. aureus (preparation A)	B0451020	Blood	BHI broth	Late stationary phase	Bacterial suspension sterilized through 0.45-μm filter
S. aureus (preparation B)	B0451020	Blood	BHI broth	Stationary phase	50% of bacterial suspension sterilized through 0.45-μm filter and 50% heat inactivated
α-Streptococcus	B1008638	Blood	Trypticase soy broth BBL with 10% inactivated horse serum and 5% Fildes enrichment BBL	Late stationary phase	10^9 CFU/mL

FBS, fetal bovine serum.

increasing severe thirst and high diuresis during the past 3 weeks. The patient had, in addition, been treated with clindamycin for a sore throat and a skin infection but disrupted the treatment 2 days before admittance because of vomiting. The patient arrived at the hospital by ambulance after being found unconscious at work. The initial Glasgow Coma Scale rating was 12. The patient was paretic in the right arm and leg but could answer questions with yes or no. Laboratory tests revealed the following: B-glucose, 33 mmol/L; a B-pH 7.29; and base excess, −12.7 mmol/L. High levels of glucose and ketones were found in the urine. The patient required 73 IU of i.v. insulin during the following 16 hours to reach a B-glucose level <10 mmol/L. A CT scan of the brain showed no bleedings and the patient received thrombolysis for a suspected brain infarction without clinical improvement. Two days after admittance, the patient's state deteriorated and he became unconscious. New CT revealed a major infarction of an area corresponding to an occlusion of the left medial cerebral artery. The patient subsequently developed edema and total brain infarction. The death of this patient was also considered to be the result of a series of complications associated with his recent T1D debut.

Animals and Operating Procedure

Healthy, male Wistar rats weighing 250 to 300 g (Taconic, Ry, Denmark) were used. To exclude strain-dependent reactions, Lewis and Sprague Dawley rats were also included in some experiments. Before the bacterial challenge, the animals were kept under standard laboratory conditions and given water and food *ad libitum*.

The animals were anesthetized by intraperitoneal injection with thiobutabarbital (Research Biochemicals International, Natick, MA). At challenge, the common bile duct was exposed through a ventral midline incision after a blunt dissection. Via the common bile duct, 0.2 mL of a suspension was injected into the pancreas. Animals were kept anesthetized for a period of 1 to 5 hours, after which they were sacrificed by heart puncture. Control animals were either sacrificed without any prior treatment or inoculated with brain heart infusion (BHI) broth without bacteria. Plasma and serum samples were assayed for aspartate transaminase, alanine aminotransferase, alkaline phosphatase, antitrypsin, orosomucoid (α_1-acid glycoprotein), haptoglobin, and C-reactive protein at the Department for Clinical Chemistry, Uppsala University Hospital. Biopsies were fixed in 4% paraformaldehyde and prepared for paraffin embedding.

Bacterial Strains and Preparation of Inocula

Four different bacterial species were used (Table 1). All strains were clinical isolates from the Department of Clinical Microbiology, Uppsala University Hospital, Uppsala, Sweden. The bacterial choice was based on a documented ability of these bacteria to translocate into the pancreas, liver, and/or gallbladder and to be leading causes of infections in these organs.[34–37] The *S. aureus* strain produced toxic shock syndrome toxin 1, the most common toxin harbored by *S. aureus* isolated from children,[38] to make it possible to study the effects of a potent exotoxin, in addition to the endotoxin of *Escherichia coli*.

The bacteria were stored at −70°C, and all cultures were initially inoculated from these frozen stocks onto blood or chocolate agar (Acumedia Manufacturers, Lansing, MI). The bacteria for pancreatic challenge were prepared by incubating at 37°C in the appropriate atmosphere for 4 to 5 hours (early stationary phase), 7 hours (stationary phase), or 15 to 20 hours (late stationary phase) according to growth curves.

The α-hemolytic streptococci were grown in trypticase soy broth BBL (Becton Dickinson, Sparks, MD) supplemented with 10% inactivated horse serum (National Veterinary Institute, Uppsala, Sweden) and 5% Fildes enrichment BBL (Becton Dickinson). All other bacterial species were inoculated into BHI broth BBL with or without 70% CMRL-1066 (ICN Biomedicals, Costa Mesa, CA) and 5% fetal bovine serum (Invitrogen, Lidingö, Sweden). Heat-killed bacteria were boiled for 15 minutes. Viable counts of all inocula were performed before challenge.

Immunohistochemistry

Consecutive 6-mm sections from the two patients with early T1D and the control donors were processed and labeled using a standard immunoperoxidase technique for paraffin section. With the exception of chromogranin A, glucagon, and insulin, all other antigens were unmasked by heat-induced epitope retrieval. Antibodies against chromogranin A (NeoMarkers, Thermo Fisher Scientific Inc, Fremont, CA) were used to identify islets. Antibodies against macrophages (CD68), granulocytes [myeloperoxidase (MPO)], and glucagon were from Dako (Glostrup, Denmark), whereas antibodies against insulin and CD3 were from BioGene (Kimbolton, UK) and Novocastra (Leica Microsystems AB, Stockholm, Sweden), respectively. Bound antibodies were visualized with Dako EnVision (Dako).

Consecutive sections from rats were processed using the same technique. Antibodies against leukosialin (CD43, all leukocytes except B cells), macrophages (ED1/CD68 and ED2/CD163), and T cells (CD3 and CD8) were from AbD Serotec (Dusseldorf, Germany); antibodies against insulin and glucagon were from Dako. The mouse antibody against insulin was from Sigma-Aldrich (St. Louis, MO). Bound antibodies were visualized using Dako EnVision or EnVision DuoFLEX Doublestain System (both Dako) and diaminobenzidine-based substrate or 3-amino, 9-ethyl-carbazole (Dako). Sections were counterstained with hematoxylin and analyzed by light microscopy.

Isolation and Culture of Human Islets

Islets of Langerhans were isolated as described previously.[39] Islet preparations were of good quality but were made available for research because of a too low total islet number for clinical transplantation. Islets were kept in culture bags (Baxter Medical AB, Kista, Sweden) with 200 mL of CMRL-1066 supplemented with 10 mmol/L HEPES, 2 mmol/L L-glutamine, 50 μg/mL of gentamicin, 20 μg/mL of ciprofloxacin (Bayer Health Care AG, Leverkusen, Germany), 10 mmol/L nicotinamide, and 10% heat-inactivated human serum (Uppsala blood bank, Uppsala, Sweden) at 37°C in 5% CO_2 and humidified air for 1 to 7 days. The culture medium was changed on day 1 and then every other day.

Human Islet Function and Cytokine Secretion in Vitro

Insulin secretion in response to glucose stimulation was assessed in a dynamic perfusion system. Twenty randomly handpicked islets were exposed to an initial baseline period with 1.7 mmol/L glucose for 36 minutes. It was followed by 16.7 mmol/L glucose for 42 minutes and a final period with 1.7 mmol/L glucose for 48 minutes. Fractions were collected at 6-minute intervals. The insulin concentration was determined by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden), and the stimulation index [(insulin released per minute during high glucose concentration)/(insulin released per minute during low glucose concentration)] was calculated. For measurements of insulin and DNA, 20 islets were randomly handpicked. Quant-iT picogreen dsDNA assay kit (Picogreen; Invitrogen) and a fluoroscan ascent FL (Thermo Electron Corp, Waltham, MA) were used for DNA measurements.

Approximately 100 to 150 handpicked islets from 7 patients were cultured in 2 mL of CMRL-1066 supplemented with 10% fetal calf serum with or without the addition of LPS (1 g/mL; InvivoGen, Toulouse, France), LTA (15 or 30 μg/mL; InvivoGen) or a combination of the two bacterial components. Supernatants were collected after 24 hours, and the concentrations of IL-8, IL-6 (R&D Systems, Oxford, UK), and monocyte chemotactic protein 1 (MCP-1) (Serotec, Oxford, UK) were measured on a Gyrolab workstation (Gyros AB, Uppsala, Sweden).

Statistical Analysis

Differences in cytokine secretion and blood works were analyzed with Wilcoxon signed rank test. $P < 0.05$ was considered statistically significant. Data are presented as mean ± SEM.

Results

Morphology of the Human Pancreas at T1D Onset

The pancreases of patients 1 and 2 had a normal macroscopic appearance. Morphologic examination of biopsy specimens retrieved from several sites before islet isolation revealed extensive ductal fibrosis and normal number and distribution of islets in both patients. However, the appearance of the β-cells within the islets differed markedly between the two patients.

Patient 1 died only a few days after the first symptoms of impaired glucose metabolism and exhibited an increased inflammation within the entire pancreas (Figure 1). The inflammatory reaction and the number of insulin-positive cells within the islets varied markedly among different lobes. Three categories of islets were observed: i) islets containing a markedly reduced number of β-cells (Figure 1, A and B, and Figure 2A), ii) islets without β-cells (Figure 1, C and D), and iii) islets with an apparent normal number of β-cells (Figure 2C). Each lobe of the pancreas contained one predominant category of islets. Slightly increased numbers of $CD3^+$ (T cells), $CD68^+$ (mono-

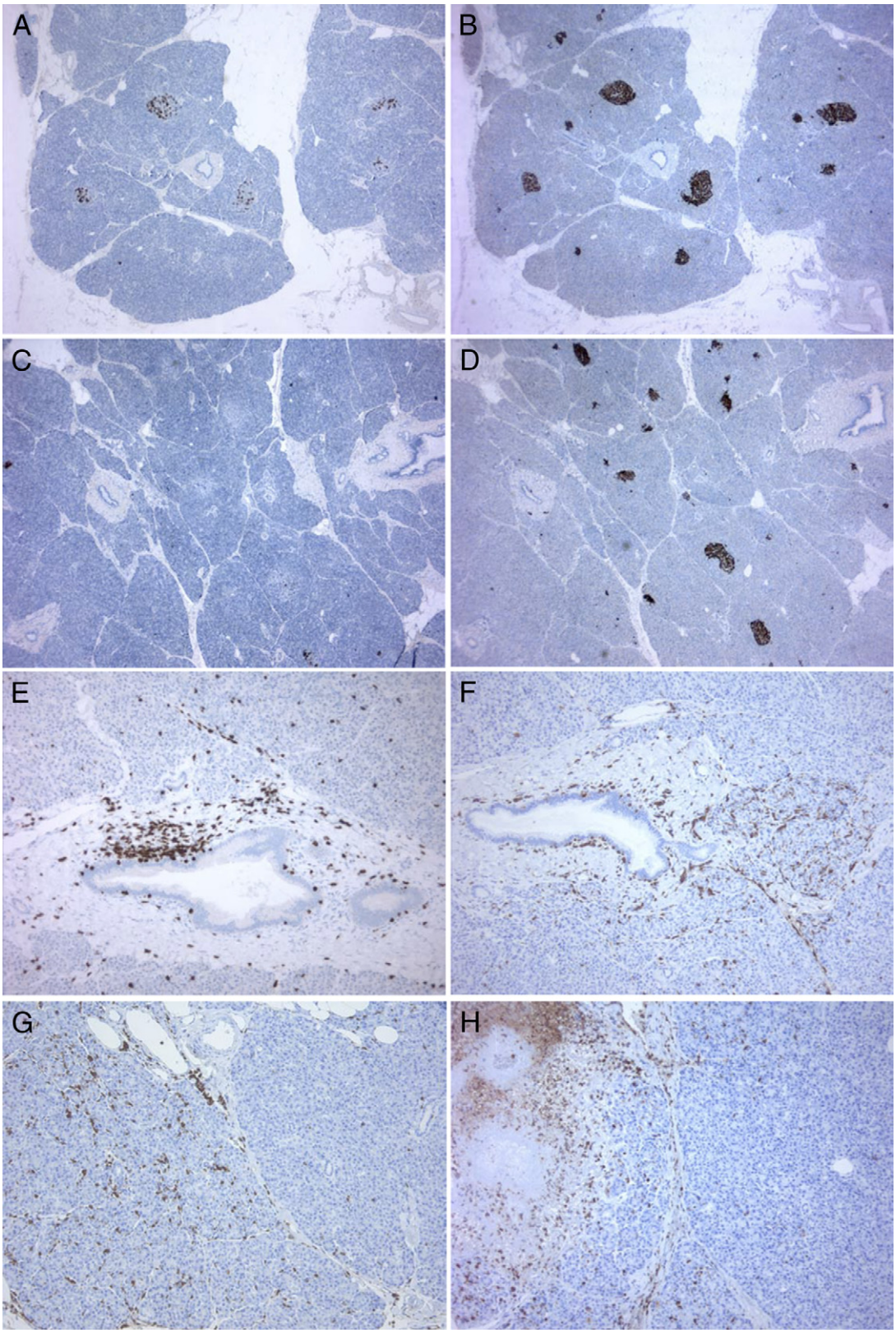

Figure 1. Immunopathologic analysis of the pancreas of patient 1. A pancreatic lobe containing islets with only a few β-cells stained for insulin (brown, **A**) and chromogranin (brown, **B**). Another lobe of the pancreas with islets with no β-cells stained for insulin (brown, **C**) and chromogranin (brown, **D**). Almost all ducts had periductal fibrosis (**A–F**). Accumulation of CD3+ (brown, **E**) and MPO^+ polymorphonuclear cells (brown, **F**) close to a duct. Migration of MPO^+ cells from ducts into surrounding exocrine parenchyma (**F** and **G**). Marked infiltration of MPO^+ polymorphonuclear cells in one pancreatic lobe but not in the adjacent lobe (**F–H**). Necrotic areas surrounding pancreatic ducts with intense inflammation of MPO^+ polymorphonuclear cells in a pancreatic lobe. The adjacent lobe has a normal appearance. Original magnification: ×25 (**D–F**) and ×100 (**E–H**).

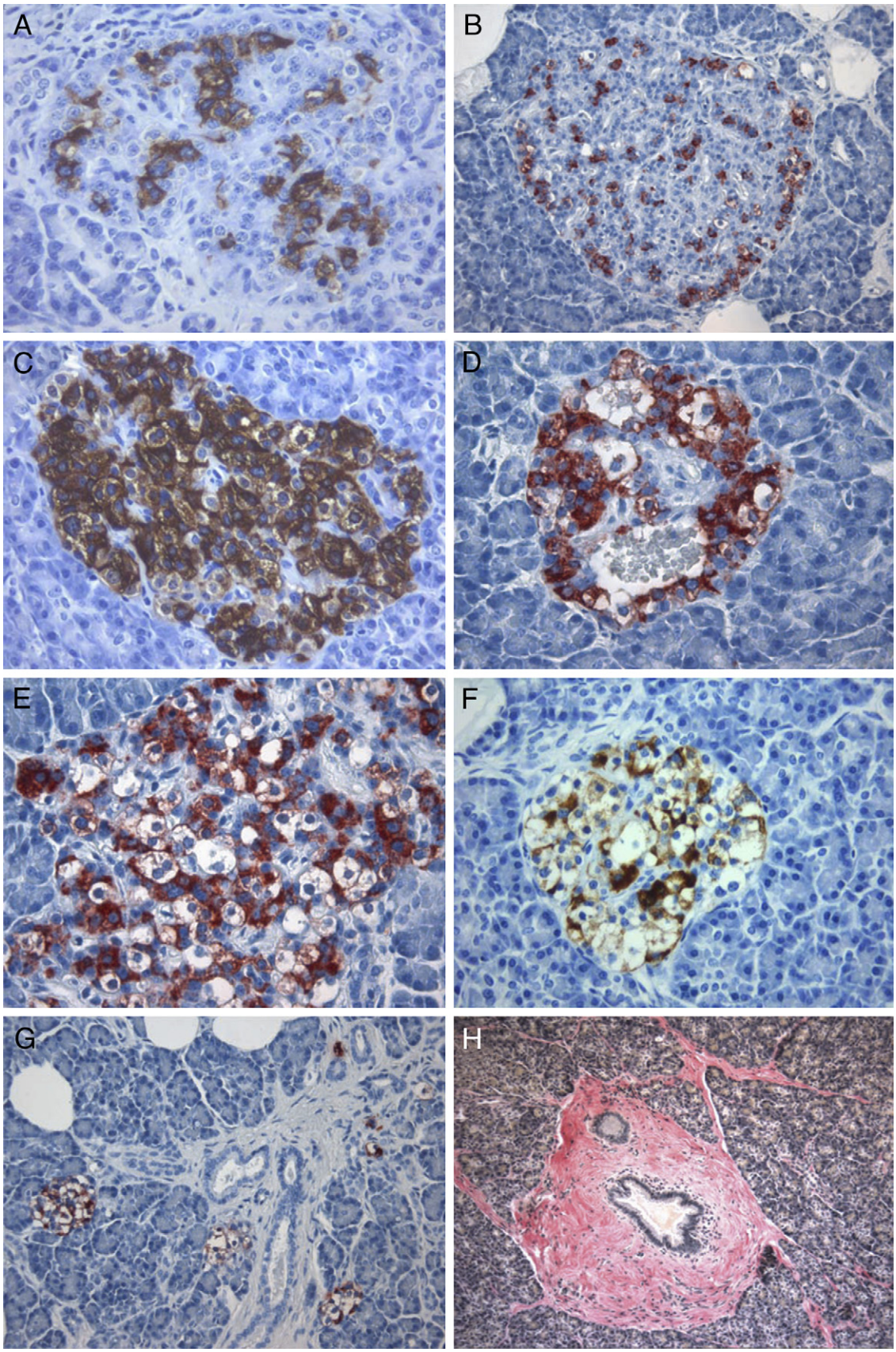

Figure 2. Immunopathologic analysis of the pancreas of patients 1 (**A**, **C**, and **H**) and 2 (**B**, **D**, **E**, **F**, and **G**). Islets with only a few remaining β-cells (brown, **A** and **B**). Islets with an apparently normal number of β-cells (brown, **C–G**). All types of islets have various degrees of hydropic degeneration and failing β-cells (brown, **A–G**). Intraislet bleeding (**D**). Periductal fibrosis (pink, Van Gieson staining). Original magnification: ×400 (**A, C–F**), ×200 (**B** and **G**), and ×100 (**H**).

cytes and macrophages), and MPO$^+$ (neutrophil granulocytes) cells were found, and they were often localized around pancreatic ducts, in septa between pancreatic lobes, but also within the pancreatic parenchyma in certain lobes (Figure 1, E–H). Occasionally, necrotic areas with intense inflammation of MPO$^+$ cells were found (Figure 1H). Clusters of immune cells were frequently observed, a few times within or close to the islets but more often in the exocrine pancreas without any apparent association to the islets. Staining for CD8 (cytotoxic T cells) and CD4 (helper T cells) revealed that most of the present CD3$^+$ cells were CD8$^+$.

In the pancreas of patient 2, who died after 3 to 4 weeks of symptoms of impaired glucose metabolism, depositions of fat were frequently observed within the exocrine parenchyma. T cells, both CD4$^+$ and CD8$^+$, CD68$^+$ macrophages, and MPO$^+$ cells were found in numbers similar or less than those observed in pancreases obtained from donors without any history of diabetes or pancreatic disease. The immune cells were scattered within the pancreatic parenchyma without any apparent accumulation in or around the islets. Most of the β-cells in almost all examined islets had signs of severe hydropic degeneration, which is characterized by a swollen cytoplasm but preserved nucleus (Figure 2, B and D–G). In some islets, intraislet hemorrhages or pronounced dilatations of the intraislet capillaries were found (Figure 2D). Most of the glucagon-positive cells had normal morphologic features, but some α-cells in the most affected islets had a swollen cytoplasm.

Macroscopic Findings in the Rat Pancreas

All animals tolerated the surgical procedure well. No macroscopic changes were observed in any other internal organ but the pancreas. Likewise, no changes in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, antitrypsin, orosomucoid (α_1-acid glycoprotein), haptoglobin, and C-reactive protein were detected in rats with bacteria installed in the pancreatic duct when compared with animals exposed to BHI without bacteria.

Four different bacterial species were used for the challenge (Table 1). Early stationary-phase bacteria induced a general infiltration of immune cells in the pancreatic parenchyma. After 2 days, these rats had developed a picture congruent with acute pancreatitis; therefore, only late stationary-phase bacteria, heat-killed bacteria, bacterial components, or mixtures thereof were used in the following experiments.

Animals challenged with the gram-negative *E. coli* exhibited regional areas of edema in their pancreases. The three gram-positive bacteria included in the study induced no or minimal macroscopic changes.

Microscopic Findings in the Rat Pancreas

After the macroscopic inspection, the pancreas was divided into three parts for microscopic examination: the head, the corpus, and the tail. Control animals exposed to BHI broth had normal morphologic findings without increased infiltration of immune cells and with intact islets.

In animals challenged with bacteria, there were regions of the pancreases that exhibited infiltration of mainly polymorphonuclear cells, with no difference among the three strains of rats used. This cellular infiltration was present as early as 1 to 2 hours after bacterial instillation (Figure 3, A and B). It increased in intensity in animals examined after 3 to 5 hours and was commonly localized to areas around the pancreatic ducts (Figure 3, C–H). Islets of Langerhans situated in the vicinity of the ducts often seemed to attract polymorphonuclear cells (Figure 4, B and C), which clustered at the border between the islet and the exocrine parenchyma or infiltrated the islets (Figure 4, D–F). Minor hemorrhages or dilated capillaries were frequently noted within the islets (Figure 3H). A large fraction of the islet cells revealed signs of severe cellular damage in the form of hydropic degeneration (Figure 3H and Figure 4, C, E, G, and H). Several islets surrounded by polymorphonuclear cells contained cells with totally destroyed cellular architecture. The exocrine cells were almost intact, but in the areas with the most intense inflammation the exocrine cells also revealed signs of degeneration.

Immunohistochemistry

Control animals instilled with BHI broth had a normal morphologic appearance and distribution of both insulin-positive and glucagon-positive cells in their pancreases. Only occasional CD68$^+$ cells were scattered within the pancreatic parenchyma. Both the frequency and distribution of these cells were the same as observed in unchallenged control animals.

The polymorphonuclear cells described herein in animals exposed to bacterial instillation in the ductal system were CD43$^+$. Most of these cells were also MPO$^+$ (neutrophil granulocytes), and the remaining cells were CD68$^+$ (monocytes and macrophages). Staining for CD3, CD8, and CD168 (activated macrophages) resulted in only a few positive cells at frequencies comparable with those recorded in control rats. Insulin and glucagon staining demonstrated that most of the islet cells with hydropic degeneration were β-cells, but some α-cells had signs of cellular damage.

Animals challenged with *E. coli* had the most intense pancreatic inflammation, often with accumulation of CD43$^+$ MPO$^+$ cells in clusters (Figures 3 and 4). In affected lobes, β-cells in both infiltrated islets and noninfiltrated islets were hydropically degenerated. If the *E. coli* was heat killed, the intensity was clearly reduced, and the generated immune reaction resembled instead that described for the gram-positive cocci. The intensity of the inflammation and the infiltration pattern did not change if a combination of *E. coli* and *Enterococcus faecalis* was used (Table 2).

Animals inoculated with enterococci or α-hemolytic streptococci induced an infiltration of CD43$^+$ MPO$^+$ cells of moderate intensity (Figures 3 and 4). The CD43$^+$ MPO$^+$ cells accumulated around ducts, in septa between the pancreatic lobes, and within some of the pancreatic lobes. The clustering tendency was, however, not as apparent as in animals inoculated with *E. coli*.

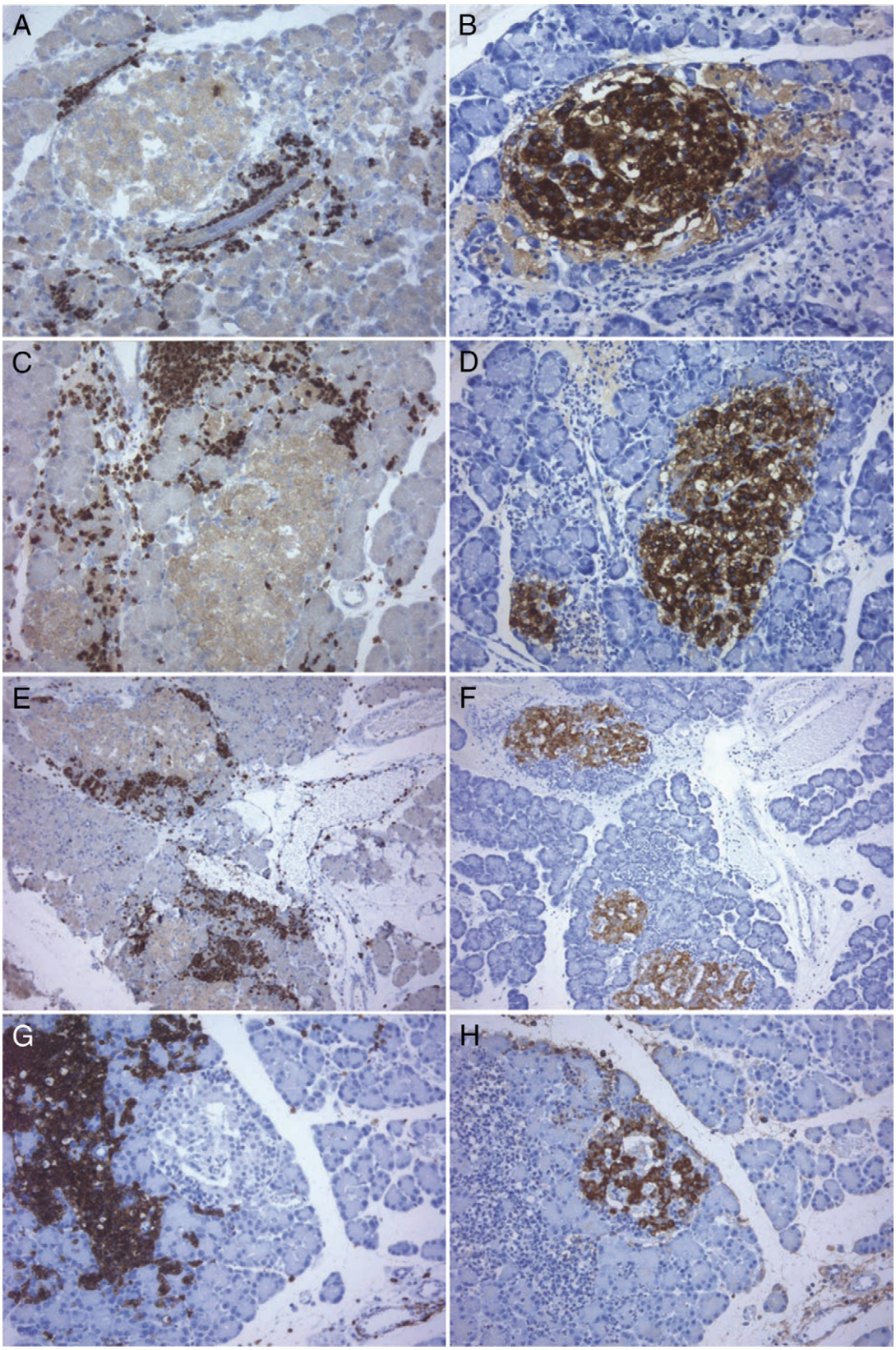

Figure 3. Progressive inflammation and islet destruction after installation of bacteria [*E. faecalis* in the late stationary phase (**A–D**) or *E. coli* in the late stationary phase (**E–H**)] in the ductal compartment of healthy rats at 1 hour (**A** and **B**), 3 hours (**C** and **D**), 5 hours (**E** and **F**), and 4.5 hours (**G** and **H**). Progressive migration of MPO^+ polymorphonuclear cells (brown, **A**, **C**, **E**, and **G**) from the ducts into the surrounding pancreatic tissue, often with accumulation around islets. Consecutive sections reveal progressive destruction of the β-cells and development of hydropic degeneration (insulin positive cells in brown, **B**, **D**, **F**, and **H**). After 4.5 hours, islets show dilatation of capillaries and intraislet bleedings (**H**). Original magnification: ×200 (**A–D** and **G–H**) and ×100 (**E** and **F**).

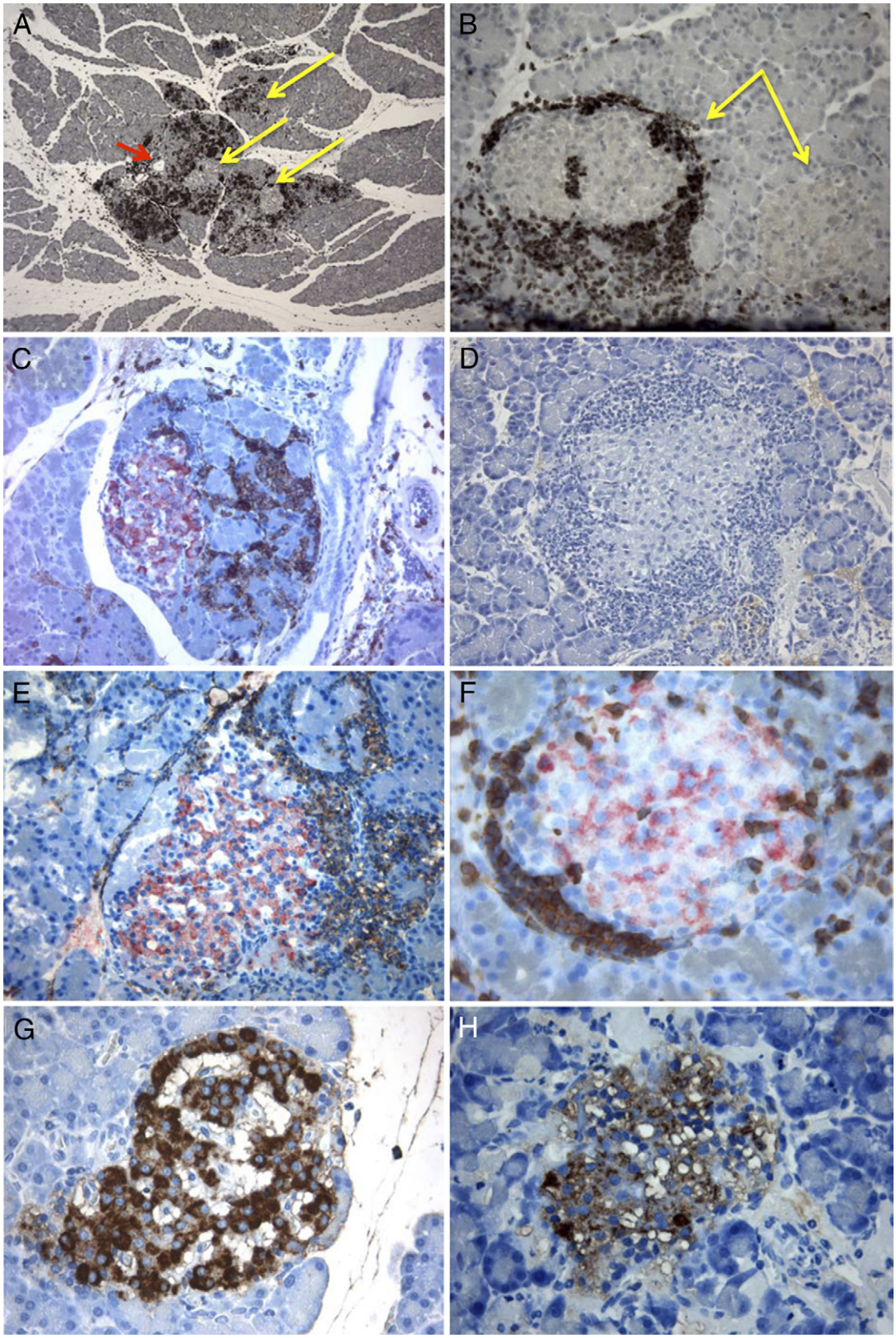

Figure 4. Islet inflammation destruction after installation (4 to 5 hours) of bacteria [*E. faecalis* in the late stationary phase (**A** and **D**) or heat-inactivated stage (**B**), *E. coli* in the late stationary phase (**C**, **E**, and **G**), α-streptococcus in the late stationary phase (**F**), or *S. aureus* suspension sterilized through 0.45-μm filter (**H**)] in the ductal compartment of healthy rats. **A:** Migration of $CD43^+$ leukocytes from a small duct (**red arrow**) and accumulation around neighboring islets (**yellow arrows**). **B:** Migration and accumulation of MPO^+ cells from a duct (lower part of the figure) to one, but not the other, of two neighboring islets (**yellow arrows**). **C:** Migration and accumulation of $CD43^+$ cells from a duct to a neighboring islet. **D:** Accumulation of polymorphonuclear cells around an islet. **E** and **F:** Accumulation and infiltration of leukocytes ($CD43^+$, brown) in an islet, with several β-cells (insulin, red) revealing hydropic degeneration. **G** and **H:** Islets with advanced hydropic degeneration and failing β-cells (insulin, brown, **A–G**). Original magnification: ×25 (**A**), ×200 (**B–E**), and ×400 (**F–H**).

Table 2. Morphologic Evaluation of Rat Pancreases Inoculated with Various Bacteria in the Ductal System

	Occasional cellular infiltration ($CD43^+$, MPO^+ cells)				Intact islet features		
Bacterial species	Periductal	Interlobular septa	Exocrine tissue	Islets	Insulin-positive cells	Glucagon-positive cells	Vasculature
E. coli (n = 12)	++ to +++	++ to +++	++ to +++ often accumulating in clusters in affected lobes	++ to +++ peri- and intraislet infiltration	Severe HD	Occasional HD	Dilatated capillaries, occasional bleedings
E. faecalis (n = 12)	+ to ++	+ to ++	+ to ++ diffusely spread within affected lobes	+ to ++ predominantly peri-islet infiltration	HD	Intact	Dilatated capillaries
E. coli and *E. faecalis* (n = 6)	++ to +++	++ to +++	++ to +++ often accumulating in clusters in affected lobes	++ to +++ peri- and intraislet infiltration	Severe HD	Occasional HD	Dilatated capillaries, occasional bleedings
S. aureus (preparation A) (n = 12)	0	0	0	0	HD*	Intact	Intact
S. aureus (preparation B) (n = 4)	0 to +	0 to +	0 to +	0 to +	HD*	Intact	Dilatated capillaries
α-Streptococcus (n = 6)	+ to ++	+ to ++	+ to ++ diffusely spread within affected lobes	+ to ++ predominantly peri-islet infiltration	HD	Intact	Dilatated capillaries

Controls (n = 9). Cellular infiltration was graded in 4 categories: 0, occasional cells, in numbers similar to untreated controls; +, low number of infiltrating cells; ++, moderate number of infiltrating cells; and +++, high number of infiltrating cells.
*More pronounced than expected when considering the amount of infiltrating cells.
HD, hydropic degeneration.

Human Islet Function and Cytokine Secretion in Vitro

Islets of Langerhans could be obtained from both patients with T1D applying a routine islet isolation procedure for the pancreases obtained from diseased organ donors. However, the total number of islets obtained from the patients with T1D was reduced, implying that only the most intact islets could be secured for *in vitro* studies. Islet insulin content was severely reduced in islets from both patients with T1D (Table 3). Glucose-stimulated insulin release in control islets had a clear biphasic response, which could not be demonstrated in islets from patients 1 or 2 (Table 3). Expression of inflammatory mediators in islets from T1D patients was in the same range as that found in control islets (Table 3).

Islets obtained from control donors that were exposed *in vitro* to LPS and LTA induced a significant increase in the secretion of IL-6 and IL-8, whereas MCP-1 needed the presence of LPS to reach significantly increased levels (Table 4).

Discussion

A new animal model was generated with an aim to reproduce the initial events of T1D. Findings obtained after inoculation of human pathogens in the pancreatic ductal compartment of healthy rats revealed marked similarities with those observed in the pancreases of patients dying at onset of T1D. Obtained results suggest that a bacterial trigger for T1D is plausible (ie, a local inflammatory reaction caused by bacteria, probably entering from the intestine to the ductal system of the pancreas, activates an innate injurious immune response, predominantly causing periductal and islet inflammation and comprising β-cell destruction) (Figure 5).

Bacteria entering through the papilla Vateri are known to occur in humans (eg, bacterial infections of the bile ducts). Enteric bacteria, especially enterococci and *E. coli,* are usually involved in acute pancreatitis.[40,41] These bacterial species, together with α-hemolytic streptococci that often colonize the human duodenum, were therefore chosen for the animal experiments. Because

Table 3. Characteristics of Isolated Islets

Islet parameter	Control islets	Patient 1	Patient 2
Stimulation index	13 ± 2	0.8*	1.9*
Insulin content, ng/ng of DNA	3.1 ± 0.5	0.092	0.26
ADP/ATP ratio	0.09 ± 0.01	0.05	0.05
Tissue factor content, pmol/μg of DNA	0.05 ± 0.009	0.031	0.067
MCP-1, pmol/μg of DNA	0.01 ± 0.002	0.02	0.019
IL-6, pmol/μg of DNA	0.003 ± 0.0007	0.01	0.0006
IL-8, pmol/μg of DNA	0.03 ± 0.006	0.04	0.1

*The total amount of insulin secreted was at nonstimulated levels.
ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Table 4. Secretion of IL-8, IL-6, and MCP-1 from Human Islets Exposed to LPS, LTA, or Both

	Control	LPS	LTA	LPS and LTA
IL-8	322 ± 104	933 ± 188*	1190 ± 500*	1136 ± 322*
IL-6	53 ± 24	121 ± 49*	162 ± 71*	119 ± 36*
MCP-1	131 ± 42	254 ± 71*	282 ± 156	273 ± 99*

Mean ± SD concentrations of IL-8, IL-6, and MCP-1 are presented in picomoles per milliliter.
*$P < 0.05$ versus control medium.

acute onset of T1D has been reported in a child with toxic shock syndrome due to *S. aureus,*[42] a toxin-producing staphylococcal strain was also included. However, because of its high virulence this strain was never used alive.

Early stationary-phase bacteria induced acute infections and necrosis. With stationary-phase or heat-killed bacteria the picture altered. A tentative initial scenario for T1D could therefore be that after contact with the pancreatic juice[29] dying or dead bacteria entrapped in the ductal system within a segment of the pancreas release toxins and cell-wall components (Figure 5) (eg, LPS and LTA). These substances trigger an innate injurious response mainly centered around affected ducts. However, bacterial components could subsequently reach neighboring islets via arterioles arising from blood vessels draining the ducts.[43] Dying β-cells that release tissue-specific antigens within a proinflammatory microenvironment provide optimal conditions for antigen-presenting cells that could trigger an acquired immune response (ie, islet specific autoimmunity, including autoantibodies[44] and in some patients also autoreactive T cells[45]). Such a T-cell–mediated response is, however, not expected to arise until after several days and is therefore beyond the scope of the present study.

Insufficiency in papilla Vateri is likely to occur more frequently during periods with rapid growth because of both the enlargement of the intestine and the pancreas. Interestingly, the HLA genotypes (HLA-DQ2/8, DQ8/0604, and DQ8/X) conferring an increased risk for T1D are also linked to increased birth weight,[46] and several studies found that children affected by T1D have increased body weight, are taller, and have higher BMI than controls.[47–51]

Cytokine-mediated toxicity has been implicated in the development of T1D. However, even if human β-cells are vulnerable to proinflammatory cytokines,[52] no plausible hypothesis has been presented to explain why these inflammatory cytokines would accumulate in the islet vicinity. Dying bacteria within the ductal system of the pancreas could provide such a mechanism. Released LPS and LTA trigger ductal epithelium and islets to release IL-6, IL-8, and MCP-1, with chemoattractive capacity to recruit mainly neutrophilic granulocytes and monocytes. These cell types were found rapidly accumulating in large numbers adjacent to the islets in affected lobules of the pancreas within only a few hours after the bacterial instillation. These infiltrating cells could induce functional impairment and death of β-cells because of their inherent low protection against proinflammatory cytokines, nitric oxide, and reactive oxygen species,[53] especially under hyperglycemic conditions.[54] An injurious innate immune response triggered by bacteria would affect only a lobe or a segment of the pancreas, whereas islets in adjacent pancreatic lobes would remain unaffected. This scenario is in agreement with findings in humans with T1D.[55] The peculiar organization of the endocrine pancreas in small clusters of cells (islets of Langerhans, apparently randomly distributed within the pancreas[56]) could possibly serve as a mechanism of protection from the develop-

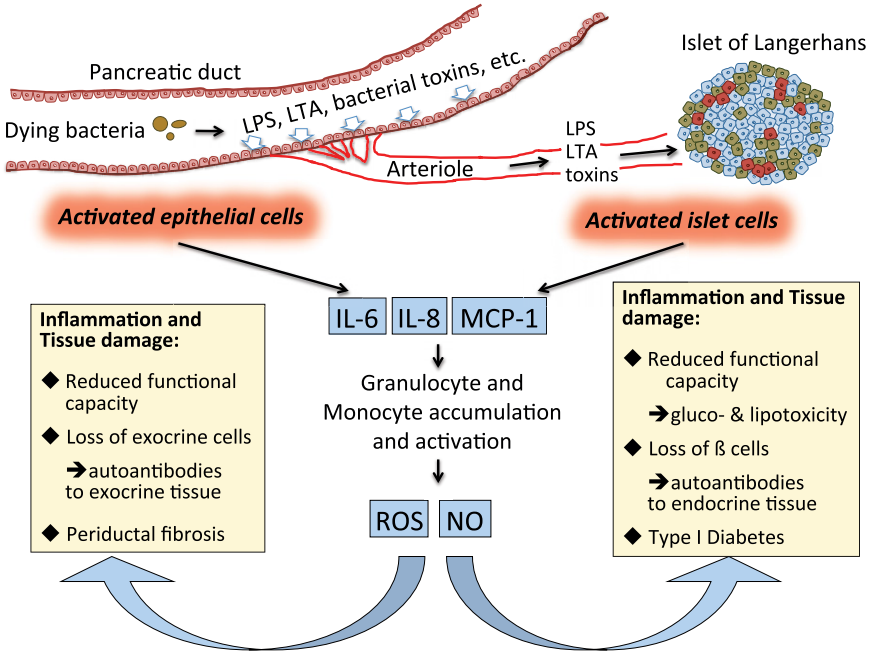

Figure 5. Bacteria unintentionally entering the ductal system of the pancreas rapidly succumb to the antibacterial effects of the pancreatic juice and release bacterial components, such as LPS, LTA, and various toxins. In response, ductal epithelial cells and islets of Langerhans receiving blood from the affected ductal area attract polymorphonuclear cells via release of IL-6, IL-8, and MCP-1. Infiltrating cells induce functional impairment and death of β-cells because of their inherent low protection against proinflammatory cytokines, nitric oxide (NO), and reactive oxygen species (ROS). Glucotoxicity and lipotoxicity, as well as autoimmunity, are superimposed and contribute to the progression into clinically overt T1D. The periductal area of the pancreas is similarly affected by the innate inflammation, leading to loss of exocrine parenchyma and periductal fibrosis.

ment of T1D via bacteria entering the defined parts of the pancreatic ductal system.

The immunopathologic findings observed in the rat model reveal marked similarities to the findings in the patient dying within only a few days of T1D (ie, an ongoing injurious inflammatory process in certain lobes of the pancreas with occasional necrotic areas densely infiltrated by neutrophilic granulocytes). Other areas of the pancreas in patient 1 had signs of preceding assaults, with increased periductal fibrosis and lobes containing islets without ongoing inflammation but with no or only a few β-cells. A few authors have reported self-resolving T1D[57] or fluctuating symptoms of T1D several months before the diagnosis of T1D.[58] These and other clinical observations led to the formulation of a model for the development of T1D[59] in which a progressive loss of β-cell mass over several years is postulated. These findings are in agreement with an injurious innate immunity reaction repeatedly occurring at certain episodes, but not with an acquired T-cell–mediated immune assault, which is expected to occur rapidly and should not vanish until all β-cells are eliminated.

In both patients who died at onset of T1D, large areas of the pancreas contained islets with an apparently normal number of β-cells, and it is difficult to envision that the remaining β-cell mass would be unable to control the glucose metabolism, implying a severe functional impairment possibly caused by an intense innate inflammation as that present in patient 1. It is tempting to speculate that the β-cells present in the pancreas of patient 2 initially were also affected by an ongoing inflammation and therefore unable to respond adequately to high glucose. When the innate immune response vanishes, some β-cells would escape this functional impairment. However, the metabolic challenge faced is insurmountable (>35 mmol/L glucose),[60] making the β-cells succumb to glucose (lipid) toxicity. This adverse process would gradually affect more and more β-cells because at each point of time the number of functionally responsive β-cells is too low to normalize blood glucose concentrations. Hydropic degeneration of the β-cells was previously described approximately 100 years ago in patients dying at onset of T1D,[61] and similar findings have been reported in a number of different conditions of prolonged periods of hyperglycemia.[62] This intracellular edema seems, therefore, to develop in response to β-cell exhaustion.

In conclusion, we present a novel animal model for the initial events triggering T1D that reveals marked similarities with the morphologic findings in patients dying at onset of T1D. Currently, only a few animal models of T1D are available.[1] The nonobese diabetic mouse and BioBreeding-diabetes prone rat predominate in the scientific literature. Although these models have generated valuable data, they are both hampered by their need for special husbandry procedures. Studies on possibly important environmental triggers of T1D are thereby excluded. The presented new model of T1D is not strain specific, and no special husbandry conditions are necessary. With longer observation periods, it may also answer questions such as why some epitopes recognized by autoreactive T cells from patients with T1D are homologous to *Streptococcus pyogenes* exotoxin A and *S. aureus* enterotoxin B[63] and how a deranged enteric flora takes part in the development of T1D and celiac disease.[64,65] Both diseases are increasing in incidence and to some extent share HLA risk genotypes (HLA-DQ2/8) and genes involved in intestinal permeability.[65] Notably, patients with celiac disease also have an enteric shift toward staphylococci and gram-negative enterobacteria.

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